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REMARKS

Claims 1, 5-22, 26 and 31-34 are pending in the application. Claims 2-4 and 35 have been canceled without prejudice and claims 23-25 and 27-29 are withdrawn.

Applicants submit these amendments with a Request for Continued Examination in view of the Advisory Action mailed January 6, 2004, in which the Examiner stated that entry of the after final amendments filed December 8, 2003, would continue to raise new rejections. Applicants previously filed an amendment on October 3, 2003, in response to a final Office Action dated June 3, 2003. The Examiner responded with an Advisory Action mailed November 19, 2003, in which he refused entry of the amendments because he alleged claim 10 would be confusing as to whether it is limiting the "selective marker" of claim 9 or the "selectable marker" of claim 1 as amended. Applicants then submitted an after final amendment on December 8, 2003, proposing to change the "selectable marker" language of claim 1 to "selective markers." The Examiner again refused entry of the amendments in the Advisory Action mailed January 6, 2004, alleging it would be confusing and unclear whether claim 10 would be limiting the "selective markers" of claim 9 or claim 1 as amended. The Examiner further alleges there is no support in the specification as filed for "selective markers" and points to page 5, lines 26-28 as support for "selectable markers."

In these amendments, Applicants have replaced the phrase "selective markers" with "selectable markers" in all the pending claims. As the Examiner points out, there is support in the specification for "selectable markers." Further, Applicants respectfully submit that one of ordinary skill in the art would appreciate that an expression vector can encode for more than one

selectable marker. Thus, it is not confusing and unclear which selectable marker claim 10 is limiting because the antecedent basis is found in both claim 9 and claim 1, as amended. Applicants respectfully request entry of these amendments and that the previously-filed amendments dated October 3, 2003 and December 8, 2003 not be entered.

I. Support for Claim Amendments

The claims were amended to more clearly define the invention. Claims 1, 21, and 26 were amended to recite that the recombinant DNA is an expression vector comprising at least one protein. The protein is encoded by a homologous or heterologous coding sequence selected from the group consisting of heparinase I, heparinase II, heparinase III, and selectable markers. Support for these amendments are found throughout the Specification, for example, on pages 11-12. Accordingly, no new matter is added by this Amendment and entry thereof is respectfully requested.

II. Objection to Claim 1

The Examiner objected to claim 1 because of informality in the language of the claim. Applicants have amended this claim as suggested by the Examiner.

III. Rejection of claims 1-22, 26 and 30-35 under 35 U.S.C. § 112, first paragraph

Claims 1-22, 26, and 30-35 are rejected under 35 U.S.C. § 112, first paragraph, as the claims allegedly contain subject matter, which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Specifically, the Examiner rejected the claims because he alleges that they "include all *Flavobacterium heparinum* host cells

transformed with any recombinant DNA construction effective to cause expression of any protein." Further, the Examiner states that "Applicants invention of a new host system for expressing DNA is recognized, however applicants new host system is not representative of any host system which employs any *Flavobacterium heparinum* host cells transformed with any recombinant DNA construction effective to cause expression of any protein."

Applicants appreciate the Examiner's recognition of their invention of a new host system for expressing DNA. Independent claims 1, 21, and 26, and claims 5-20, 22, and 31-34 dependent thereon, were amended to recite a *Flavobacterium heparinum* host transformed with an expression vector effective to cause expression of at least one of heparinase I, heparinase II, heparinase III, or a selectable marker. Claims 2-4 and 35 have been canceled. Applicants respectfully submit that the claims fully comply with the guidelines for written description required by 35 U.S.C. § 112, first paragraph. It is clear from the disclosure in the Specification that Applicants are in possession of the claimed invention, a new *Flavobacterium heparinum* host system for expressing heparinase enzymes or selectable markers using expression vectors. Examples 1 and 2 on pages 8-9 of the Specification describe construction of expression vectors for expression of both homologous genes and heterologous genes in the *Flavobacterium heparinum* host cell. Example 4 on page 11 of the Specification describes the expression of heparinase I, heparinase II, heparinase III, and selectable markers by the *F. heparinum* host transformed with the expression vectors.

The Examiner has further rejected claims 1-22, 26, and 30-35 under 35 U.S.C. § 112, first paragraph, for lack of enabling disclosure. The Examiner asserts that the Specification does not reasonably provide an enabling disclosure for *Flavobacterium heparinum* transformed with any

vector. Applicants respectfully traverse the rejection. Applicants have provided a complete and enabling disclosure of their invention, as recognized by the Examiner to be a new host system for expressing DNA. As set forth above, the Specification and the Examples teach and demonstrate to one having skill in the art how to make the expression vectors of the present invention and how to obtain expression in the *F. heparinum* host. Nevertheless, Applicants have amended the claims to recite a *Flavobacterium heparinum* host transformed with an expression vector effective to cause expression of at least one of heparinase I, heparinase II, heparinase III, or selectable markers. Applicants submit that these amendments obviate the instant rejection for the reasons discussed in the foregoing paragraph. Withdrawal of the rejection of claims 1, 5-22, 26, and 30-34 under 35 U.S.C. § 112, first paragraph, is respectfully requested.

IV. Rejection of claims 1-11, 13-21, 26, and 30-35 under 35 U.S.C. § 103(a)

Claims 1-11, 13-21, 26, and 30-35 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Zimmerman et al. (WO 96/01894, January 25, 1996) and McBride et al. (Applied and Environmental Microbiology, Vol. 62, No. 8, pages 3017-3022, August 1996). Zimmerman et al. is relied on for teaching the culturing of *Flavobacterium heparinum* and the isolation and cloning of the genes encoding the enzymes, chondroitinase AC and chondroitinase B, from *Flavobacterium heparinum*. Zimmerman et al. is further relied upon to teach that the cloned genes can be used in conjunction with suitable expression systems to produce enzymes in *Flavobacterium*, for example, under the control of overexpression promoters, or in organisms other than *Flavobacterium*. McBride et al. is relied on for using the tn4351 transposon to introduce heterologous DNA into *Flavobacterium meningosepticum*, activities which the Examiner alleges provides a reasonable expectation of success for the *Flavobacterium*

heparinum expression system as suggested by Zimmerman et al. The Examiner concludes that one of ordinary skill in the art at the time of the filing would have been motivated to express the genes encoding the *Flavobacterium heparinum* enzymes, chondroitinase AC and chondroitinase B, in *Flavobacterium heparinum* under the control of an overexpression promoter in a suitable expression system, such as the tn4351 transposon DNA, as taught by McBride at al. and suggested by Zimmerman et al. Applicants respectfully traverse this rejection.

Independent claims 1, 21, and 26, and claims 5-11, 13-20, and 30-34 dependent thereon, as amended, recite a *Flavobacterium heparinum* host transformed with an expression vector effective to cause expression of at least one of heparinase I, heparinase II, heparinase III, or a selectable marker. Claims 2-4 and 35 have been canceled. Applicants respectfully submit that these claims are not rendered obvious by the combination of Zimmerman and McBride references. In order to establish a *prima facie* case of obviousness under 103(a), all claim limitations must be taught or suggested by the prior art. The use of expression vectors for expression of heparinase I, heparinase II, heparinase III, or a selectable marker in a *F. heparinum* host is not taught or suggested by either Zimmerman or McBride, alone or in combination. The Examiner alleges that Zimmerman and McBride would have motivated one of ordinary skill in the art at the time of filing to express the genes encoding the *Flavobacterium heparinum* enzymes, chondroitinase AC and chondroitinase B, in *Flavobacterium heparinum* under the control of an overexpression promoter in a suitable expression system, such as the tn4351 transposon DNA. What Applicants are teaching is the expression of homologous or heterologous gene sequences encoding at least one of heparinase I, heparinase II, heparinase III, or a selectable marker in *Flavobacterium heparinum* in an expression system using expression

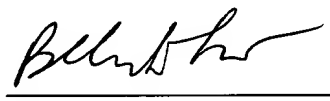
vectors, not the tn4351 transposon DNA. In fact, the inventors attempted to transfer DNA into *F. heparinum* via a transposon but were not able to obtain DNA integration, as shown in the attached Su et al., *Microbiology* 147: 581-589 (2001) reference. In particular, the authors state that previous attempts to use transposons into *F. heparinum* failed. *See* page 585, col. 2, first full paragraph. Accordingly, the combination of Zimmerman and McBride cannot establish a *prima facie* case of obviousness.

Applicants submit that the claimed invention is not rendered obvious by Zimmerman and McBride because the references do not teach or suggest all the limitations of the claimed invention. Withdrawal of the rejection of claims 1, 5-11, 13-21, 26 and 30-34 under 35 U.S.C. § 103(a) is respectfully requested.

IX. CONCLUSION

In view of the foregoing remarks, Applicants believe that the application is in condition for allowance. However, if the Examiner disagrees, he is encouraged to call the undersigned at the number listed below in order to expedite the prosecution of this application.

Respectfully submitted,



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Development f a genetic system for the transfer of DNA into *Flavobacterium heparinum*

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***Flavobacterium heparinum* (now *Pedobacter heparinus*) is a Gram-negative soil bacterium which can produce yellow pigments. It synthesizes five enzymes that degrade glycosoaminoglycan molecules. The study of this unique bacterium has been limited by the absence of a genetic manipulation system. In this paper, the construction of a conjugation/integration plasmid system and a broad-host-range plasmid, both of which contain a *F. heparinum* functional selective marker created by placing the trimethoprim resistance gene, *dhfrII*, under the control of the *hepA* regulatory region is described. Both plasmids were introduced into *F. heparinum* by conjugation and/or electroporation, and trimethoprim resistant colonies were obtained. Fifty electroporants were obtained per microgram covalently closed circular plasmid DNA. The existence of integrated plasmid DNA was confirmed by Southern hybridization and PCR. The existence of a derivative of the broad-host-range plasmid pBBR1 in *F. heparinum* was demonstrated by plasmid digestion and Southern hybridization, and by transformation of *Escherichia coli*.**

Keywords: *Flavobacterium heparinum*, integration vector, plasmid replication, heterologous DNA transfer

INTRODUCTION

Flavobacterium heparinum is a non-pathogenic soil bacterium isolated by Payza & Korn (1956). The bacterium was described as a Gram-negative, strictly aerobic, nonsporulating rod, which produces yellow pigments when grown on agar (Steyn *et al.*, 1998). The study of this bacterium is of interest as it can degrade heparin and sulfated acidic mucoheteropolysaccharides from various animal tissues and utilize them as the sole sources of carbon, nitrogen and energy (Linhardt *et al.*, 1986; Gu *et al.*, 1995). Earlier studies revealed that it synthesizes five glycosoaminoglycan-degrading enzymes: heparinases I, II and III (Yang *et al.*, 1985; Zimmermann *et al.*, 1990; Lohse & Linhardt, 1992), and chondroitinases AC and B (Yamagata *et al.*, 1968; Michelacci & Dietrich, 1975; Gu *et al.*, 1995), which depolymerize heparin, heparan sulfate and chondroitin sulfates.

The taxonomy of *F. heparinum* is not clear. It has been reclassified as *Cytophaga heparinum* by Christensen (1980), *Sphingobacterium heparinum* by Takeuchi & Yokota (1992) and lately, as *Pedobacter heparinus* by Steyn *et al.* (1998). Early 16S rRNA sequence analysis suggests that *F. heparinum* is more closely related to *Bacteroides fragilis* than to other species, such as *Bacillus subtilis*, *Escherichia coli*, *Desulfovibrio desulfuricans* and *Agrobacterium tumefaciens* (Weisburg *et al.*, 1985), even though these two bacteria differ in terms of their physiological characteristics and natural habitat. It is well known that the designation of *Flavobacterium-Cytophaga-Bacteroides* genera is permissive. In view of the unclear taxonomy, we have chosen to use the name *Flavobacterium heparinum* in this paper.

We wanted to develop a method for transfer of DNA

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Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Gm, gentamicin; HT, trimethoprim-resistance gene (cassette); Km, kanamycin; Tc, tetracycline; Tp, trimethoprim.

The GenBank accession number for the sequence reported in this paper is AF221716.

into *F. heparinum*. Gram-negative bacterial broad-host-range plasmids belonging to several incompatibility groups have been widely used for the genetic manipulation of many micro-organisms. IncP group plasmids, such as RP4, R751 and their derivatives, have been successfully transferred and stably inherited among a large number of Gram-negative bacteria (Thomas & Helinski, 1989). IncQ group plasmids, such as RSF1010, are of small size, high copy number and have a wide host range. Such plasmids have been shown to replicate in most Gram-negative bacteria (Frey & Bagdasarian, 1989) and in some Gram-positive actinomycetes such as *Mycobacterium smegmatis* and *Streptomyces lividans* (Gormley & Davies, 1991). Furthermore, *Bacteroides* plasmid pB8-51 was shown to replicate in *Prevotella ruminicola* (Shoemaker *et al.*, 1991) and *Porphyromonas gingivalis* (Maley *et al.*, 1992), and its transposon, Tn*4351*, has been used for the genetic manipulation of bacteria which are members of *Flavobacterium*, *Cytophaga*, *Flexibacter* and *Sporocytophaga* species (McBride & Baker, 1996; McBride & Kempf, 1996). However, several commonly used broad-host-range Gram-negative bacterial plasmids from different incompatibility groups could not replicate in several *Bacteroides* strains (Shoemaker *et al.*, 1986), *Pre. ruminicola* (Shoemaker *et al.*, 1991), *Por. gingivalis* (Maley *et al.*, 1992) and *Cytophaga johnsonae* (McBride & Kempf, 1996), and possibly many other bacteria of which we are unaware.

Plasmid pBBR1 (Antonie & Locht, 1992), from the Gram-negative bacterium *Bordetella bronchiseptica*, has been characterized as a small, high-copy-number plasmid with the ability to replicate in a broad range of Gram-negative bacteria including *Acetobacter*, *Alcaligenes*, *Bartonella*, *Bordetella*, *Burkholderia*, *Caulobacter*, *Escherichia*, *Paracoccus*, *Pseudomonas*, *Rhizobium*, *Rhodobacter*, *Salmonella*, *Vibrio* and *Xanthomonas* (Antonie & Locht, 1992; Elizer *et al.*, 1995; Kovach *et al.*, 1995; DeShazer & Woods, 1996). It has been shown not to belong to the IncP, IncQ or IncW groups of broad-host-range plasmids (Antonie & Locht, 1992). DNA sequence analysis showed it to be a 2.6 kbp plasmid with two major ORFs, *rep* and *mob*, encoding proteins responsible for plasmid replication and mobilization, respectively.

In this paper, we report the development of a genetic manipulation system for *F. heparinum*. A conjugative/integrative plasmid with a *F. heparinum* functional selective marker for the transfer of heterologous DNA into *F. heparinum* has been constructed. It was demonstrated that this plasmid integrates into the *F. heparinum* chromosome. In addition, a derivative of the broad-host-range plasmid pBBR1Cm with a *F. heparinum* functional selective marker has been shown to replicate in *F. heparinum*.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria broth (LB) at 37 °C, unless stated otherwise. Minimal medium (FM) supplemented with either 1% semipurified heparin (Celsus Laboratories) (FMH medium) or 0·4% glucose (FMG medium) was used for the growth of *F. heparinum* as described by Zimmermann *et al.* (1990). *F. heparinum* cultures or plates were incubated at 23 °C, unless stated otherwise. To select for *F. heparinum* transconjugant or transformant strains, antibiotics were added at the following concentrations (μg ml^{-1}): tetracycline (Tc), 50; chloramphenicol (Cm), 100; trimethoprim (Tp), 600 and erythromycin (Em), 300. For the counter-selection, gentamicin (Gm), 60 and kanamycin (Km), 100 were used. *E. coli* transformants were selected by addition of ampicillin (Ap), Tc, Cm and Tp at concentrations of 100, 20, 25 and 10 μg ml^{-1}, respectively.

Analysis of *F. heparinum* for antibiotic resistance. *F. heparinum* cells were plated on either FMH or FMG containing various concentrations of the antibiotics tested. The plates were incubated at either 30 or 23 °C, for 5 or 7 d, respectively.

Conjugation. *E. coli* strain S17-1 was used for plasmid conjugative transfer. Donor *E. coli* strains containing the plasmids were grown to mid-exponential phase in LB medium. Recipient *F. heparinum* was grown in FMH medium for 2 d, diluted fivefold in the same medium and grown for an additional 5 h. Aliquots of 0·1 ml donor and 1·0 ml recipient cells were mixed, concentrated and plated on a 2·5 cm diameter (0·45 μM pore size) HA filter (Millipore; catalogue no. HAWG 02500) which was placed on FMH/LB plates. After incubation overnight at 30 °C, the cells were harvested from the filters in 1 ml FM medium and plated on selective MHGKT (MH medium containing Gm, Km and Tp) plates. After 7 d incubation at 23 °C, antibiotic-resistant colonies appeared and were confirmed by streaking for single colonies on similar plates.

Molecular biology techniques. Isolation of chromosomal DNA, cloning and DNA manipulation techniques were performed as described by Sambrook *et al.* (1989). T4 DNA ligase, DNA polymerase I Klenow fragment and restriction endonucleases were purchased from New England Biolabs. DNA fragments destined for ligation were first separated by agarose gel electrophoresis and the DNA was extracted from the agarose with a GeneClean I kit (Bio101).

Electroporation. DNA was introduced into *E. coli* by electroporation using a Gene Pulser electroporator (Bio-Rad) as described by the manufacturer. DNA from *E. coli* transformants was isolated for restriction analysis using the RPM kit (Bio101). Electrocompetent *F. heparinum* cells were prepared by growing them to exponential phase in FMH medium. The cells were harvested, washed once with ice-cold water, twice with ice-cold 10% (v/v) glycerol and concentrated 20-fold in 10% (v/v) glycerol solution. The cells were aliquoted and either used immediately or stored at −70 °C. Plasmid DNA (1 μg) was added to 50 μl cells in a 2 mm-electrode-gapped cuvette (Bio-Rad) and subjected to a 2·5 kV electrical pulse with a capacitance of 25 μF and an external resistance of 400 Ω. Electroporated cells were immediately diluted into 1 ml LB medium with 1% heparin and incubated for 5 h at 23 °C. The cells were then harvested and plated on MHGKT plates. Antibiotic-resistant colonies normally appeared within 7 d and were confirmed by streaking for single colonies on MHGKT plates.

DNA hybridization. DNA was prepared for Southern blotting (Southern, 1975) by digestion with appropriate restriction endonucleases, separated by electrophoresis on a 0·8%

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and relevant characteristics	Source or reference
Strains		
E. coli		
S17-1	*hsdR17* (r_k^- m_k^-) *recA* RP4-2(Tc^r::Mu-Km^r::Tn*7* Str^r)	Simon *et al.* (1983)
XL-1 Blue		Bullock *et al.* (1987)
F. heparinum		
ATCC 13125	Wild-type	ATCC
FIBX1	pIBXF1 integrated into *F. heparinum* chromosome	This study
FIBX2	*F. heparinum* pIBXF2 plasmid transconjugant	This study
Plasmids		
R751	IncP, Tp^r	Meyer & Shapiro (1980)
pSUP106	Inc Q, Cm^r Tc^r	Priefer *et al.* (1985)
pBBR1Cm	Broad-host-range plasmid from *B. bronchiseptica*, Cm^r	Antonie & Locht (1992)
pNJR12	IncQ, *E. coli–Bacteroides* shuttle vector, Km^{r*} Tc^r†	Maley *et al.* (1992)
pCP11	*E. coli–C. johnsonae* shuttle plasmid, Ap^{r*} Em^r†	McBride & Kempf (1996)
pTZ/PC	PCR DNA fragment cloning vector, Ap^r	Su *et al.* (1996)
pUC21	Ap^r	Vieira & Messing (1991)
pUC13-*oriT*	Ap^r, *oriT*	Wang *et al.* (1995)
pIB17	2·2 kb *Bam*HI–*Hin*dIII *hepA* DNA fragment in pUC21, Ap^r	This study
pIB18	830 bp *hepA* upstream sequence in pTZ/PC, Ap^r	This study
pIB19	200 bp *dhfrII* gene in pTZ/PC, Ap^r	This study
pIB20	HT cassette in pTZ/PC, Ap^r	This study
pIB21	HT cassette in pUC13-*oriT*, Ap^r	This study
pIB22	HT cassette in pUC21, Ap^r	This study
pIBXF1	A 10 kbp *Hin*dIII DNA fragment in pIB21, Ap^{r*} Tp^r‡	This study
pIBXF2	pBBR1Cm derivative with *mob*, Ap^{r*} Tp^r‡	This study
pSUP106HT	HT cassette in pSUP106, Cm^{r*} Tp^r†	This study
pNJR12HT	HT cassette in pNJR12, Km^{r*} Tc^r† Tp^r‡	This study
pCP11HT	HT cassette in pCp11, Ap^{r*} Em^r† Tp^r‡	This study

* Expressed in *E. coli*.

† Expressed in *Bacteroides* spp. and *C. johnsonae*.

‡ Expressed in *F. heparinum*.

agarose gel and transferred to a nylon membrane (Hybond-N; Amersham). The probes were labelled with [α-^{32}P]dCTP, using the Rediprime II kit (Amersham Pharmacia Biotech). Hybridization was performed with Rapid-hyb buffer (Amersham Pharmacia Biotech) as described by the manufacturer.

DNA amplification. Oligonucleotide primer synthesis, preparation of *F. heparinum* genomic DNA or plasmid DNA for use as template and analysis of PCR-generated products were all conducted as previously described (Su *et al.*, 1996). PCR amplification was performed according to Mullis *et al.* (1986) with the modifications described previously (Su *et al.*, 1996). The annealing temperature was 55 °C.

DNA sequence analysis. DNA sequences were determined by the dideoxy chain termination method of Sanger *et al.* (1977), using an ALF automated DNA sequencer (Amersham Pharmacia Biotech). Sequencing reactions were prepared with the Pharmacia Auto Read fluorescent labelling kit (Amersham Pharmacia Biotech). The DNA sequence of the *hepA* upstream region in plasmid pIB17, constructed by cloning a gel-purified 2·2 kbp *Bam*HI–*Hin*dIII DNA fragment containing the *hepA* gene from plasmid pIB10 into pUC21 was determined by the primer-walking method.

Insertion of the Tp-resistance gene cassette (HT) into various plasmids. Plasmids pSUP106HT and pNJR12HT were constructed by inserting a gel-purified 1·1 kbp *Bam*HI–*Hin*dIII DNA fragment from pIB21 into the corresponding sites of pSUP106 and pNJR12, respectively. pCP11HT was constructed by cloning a gel-purified 1·1 kbp *Spe*I DNA fragment from pIB22 (formed by cloning a gel-purified 1·1 kbp *Eco*RI DNA fragment from pIB21 into pUC21) into the unique *Xba*I site of pCP11.

Plasmid pIBXF1 construction. Primers OH1-1 (5′-GGGAATTCTTTCGCCGGTGAAAGGATTG-3′) and OH1-2 (5′-GGCATATGTCTTTAGTTTTTATTGG-3′) were used to amplify an 830 bp PCR fragment, corresponding to the upstream region of *hepA*, from plasmid pIB10. Primers RT-1 (5′-GGCATATGGACCAACACAACAATGGA-3′) and RT-2 (5′-TTGAATTCTTAGGCCACGCGTTCAAGCG-3′) generated a 250 bp PCR fragment corresponding to *dhfrII* from plasmid R751. The 830 and 250 bp fragments were cloned into plasmid pTZ/PC to create plasmids pIB18 and

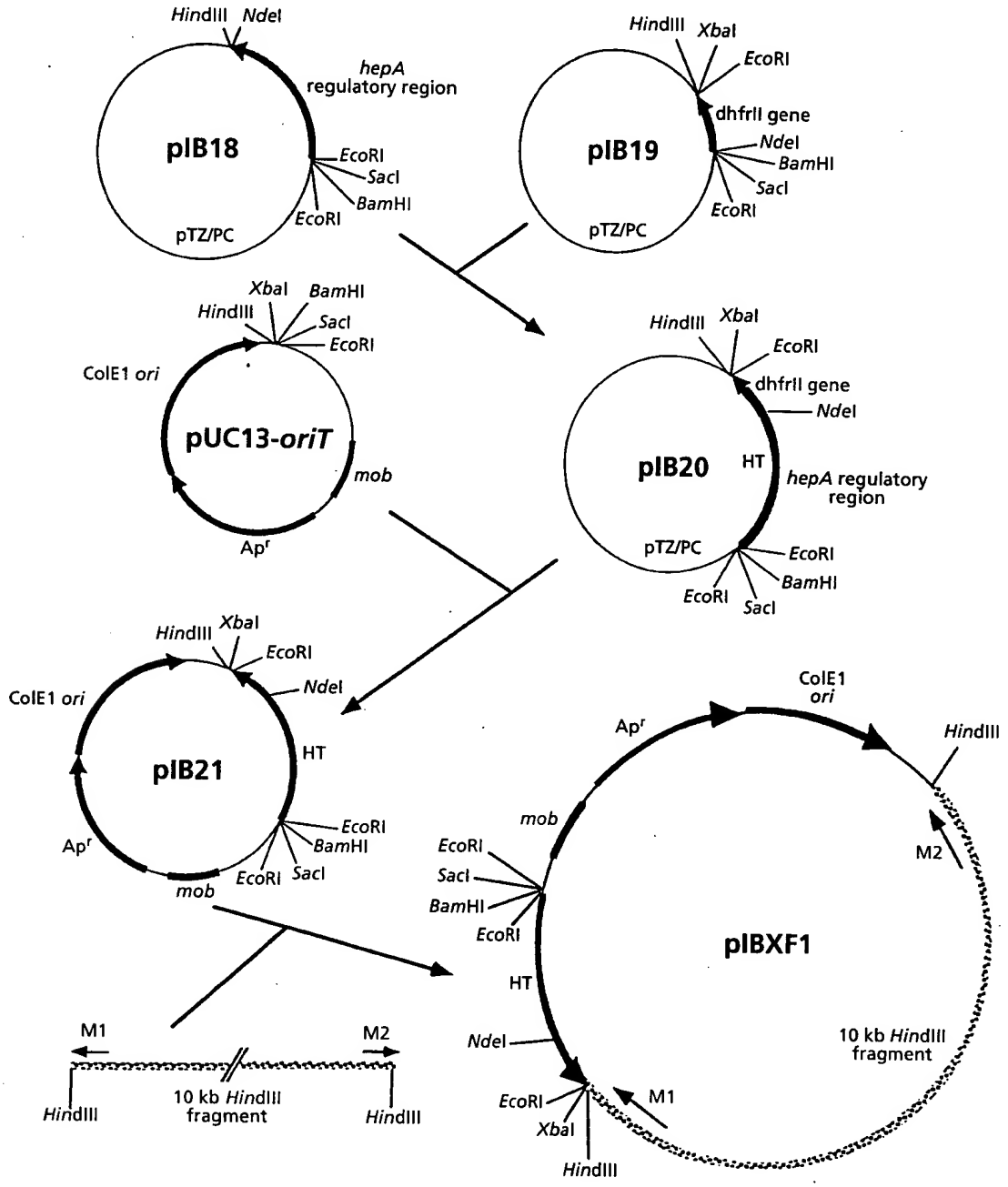

Fig. 1. pIBXF1 construction. Solid black lines indicate coding regions; arrows, orientation of the genes; thin lines, plasmid sequence (pTZ/PC or pUC13-*oriT*); speckled line, *F. heparinum* chromosomal DNA. Two primers, M1 and M2, used in Fig. 3 are also shown as small arrows.

pIB19, respectively (as shown in Fig. 1). Both inserts were confirmed by DNA sequence analysis. A gel-purified 250 bp *NdeI–HindIII* DNA fragment from pIB19 was inserted into the corresponding sites of pIB18, to yield plasmid pIB20. pIB21 was constructed by inserting a gel-purified 1·1 kbp *BamHI–HindIII* DNA fragment from pIB20 into pUC13-*oriT*. Finally, a randomly chosen 10 kbp DNA fragment from *HindIII*-digested *F. heparinum* chromosomal DNA was cloned into the unique *HindIII* site of plasmid pIB20 to yield plasmid pIBXF1.

Plasmid pIBXF2 construction. Primers OIB20-1 (5′-GGCT-CGAGTAAACTTGGTCTGACA-3′) and OIB20-2 (5′-CC-CTCGAGCGGATAACAATTTCACAC-3′), were used to amplify a 3·0 kbp PCR fragment from pIB21 containing *mob*, Ap^r and the HT cassette. This 3·0 kbp gel-purified PCR product and the 4·0 kbp *EcoRI*-linearized pBBR1Cm vector were both treated with DNA polymerase I Klenow fragment to create blunt ends and ligated. The mixture was transformed into *E. coli* XL-1 Blue and selected for Ap^r colonies. From

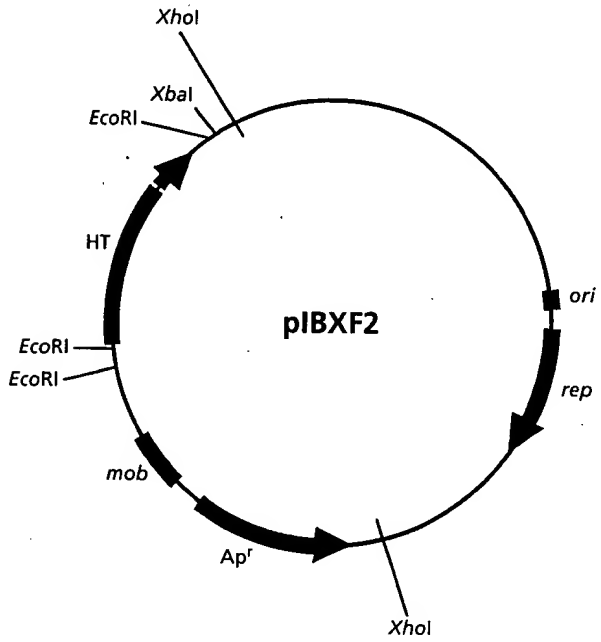

Fig. 2. Map of pIBXF2. DNA to the right and left of the *Xho*I site is from pBBR1Cm and pIB21, respectively. Thick lines represent the indicated coding regions. Arrows indicate the orientation of the genes.

these, pIBXF2 was purified and confirmed by restriction analysis (Fig. 2).

Purification of pIBXF2 from *F. heparinum*. For small-scale plasmid purification, strain FIBX2 was grown in 5 ml MHGKT medium for 2 d and DNA was isolated using the RPM kit (Bio101). Alternatively, strain FIBX2 was grown in 30 ml MHGKT medium overnight, subcultured into 500 ml of the same medium and grown for an additional 24 h. Plasmid DNA was isolated with a Qiagen-tip 500, according to the manufacturer's recommendations for low-copy-number-plasmid purification. The DNA was resuspended in 150 µl dH_2O. Fifteen microlitres of the sample were used for the experiment shown in Fig. 4.

RESULTS

Identification of antibiotics for selection and counter selection in *F. heparinum*

The antibiotic-resistance spectrum of *F. heparinum* has been briefly described by Steyn *et al.* (1998). However, these data were insufficient for the identification of selective (sensitive) or counter-selective (resistant) antibiotic markers. Experiments were designed and conducted to determine which antibiotics could be used for the genetic manipulation of *F. heparinum*. Antibiotic resistance was defined as no decrease in the number of colonies growing on medium in the presence or absence of antibiotics at a given concentration, after incubation at 23 °C for 7 d or 30 °C for 5 d. A selective marker was defined as one which restricted growth to none or very few colonies after plating in excess of 10^{10} *F. heparinum* cells on antibiotic-containing medium after incubation at 23 °C for 7 d or 30 °C for 5 d. The results showed that *F. heparinum* was resistant to Gm and Km at 60 and 100 µg ml^{-1}, respectively, and sensitive to Cm, Em, Tc and Tp at 100, 300, 50 and 600 µg ml^{-1}, respectively.

Construction of a conjugation/integration plasmid for *F. heparinum*

To date, no publication has described any DNA transfer into *F. heparinum*. Our previous attempts to transfer broad-host-range plasmids and transposons into this micro-organism failed (data not shown). Therefore, a new system was developed based on the essential elements of DNA transfer, maintenance and selection. An *E. coli* conjugative plasmid and a randomly cloned DNA fragment from the *F. heparinum* chromosome would allow for both DNA transfer and stable DNA maintenance through homologous recombination. Obtaining a functional selective marker in *F. heparinum* was the main hurdle in the creation of this system.

Thus far, no selective marker has been demonstrated to be functional in *F. heparinum*. To ensure that a selective marker, e.g. an antibiotic-resistance gene, would be expressed in *F. heparinum*, a hybrid gene needed to be assembled which would consist of the desired antibiotic-resistance gene placed under the control of a *F. heparinum* regulatory region. Few genes from *F. heparinum* have been studied at the molecular level. DNA sequences are available only for the five glycosaminoglycan lyase genes *hepA*, *B*, *C* and *cslA*, *B* (Sasisekharan *et al.*, 1993; Su *et al.*, 1996; Tkalec *et al.*, 2000). Among them, it appeared that *hepA* was expressed at the highest level when grown in heparin-only medium (Lohse & Linhardt, 1992). Therefore, the *hepA* regulatory region was chosen to construct a hybrid antibiotic-resistance gene, which would allow for selection on heparin-only medium. A small region of 170 bp, 5′ of the *hepA* start codon (upstream region), had been previously identified (Sasisekharan *et al.*, 1993). Within this sequence, no typical prokaryotic promoter elements were found. It was probable that this 170 bp *hepA* upstream region did not contain all the necessary elements that controlled *hepA* gene expression. Further sequence analysis of an 830 bp *hepA* upstream region in plasmid pIB17 was performed (GenBank accession no. AF221716). Although it did not reveal typical promoter elements, a partial putative ORF was identified, which translated in a direction opposite to, and 325 bp away from, the start codon of *hepA*. This suggested that the promoter region for *hepA* was probably located within this 325 bp region. To ensure that all the regulatory elements were included, the 830 bp *hepA* upstream region was used to create a hybrid antibiotic-resistance gene cassette.

The Tp-resistance gene, *dhfrII*, from plasmid R751 (Meyer & Shapiro, 1980), was chosen for the hybrid antibiotic-resistance cassette construction. This gene has been shown to confer very high levels of Tp

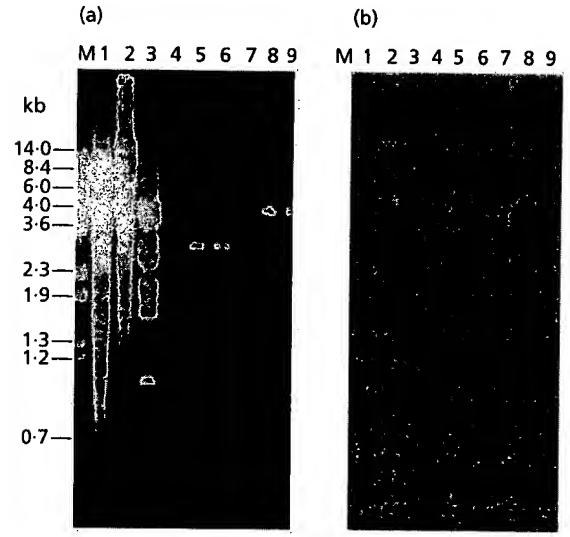

Fig. 3. Demonstration of pIBXF1 insertion into *F. heparinum* chromosomal DNA. (a) Ethidium bromide stained agarose gel. (b) Autoradiograph of the gel from (a) probed with pIB21. Lanes: M, DNA molecular mass markers (in kb) are indicated on the left; 1, *F. heparinum* chromosomal DNA digested with *Hin*dIII/*Eco*RI; 2, FIBX1 chromosomal DNA digested with *Hin*dIII/*Eco*RI; 3, pIB21 digested with *Eco*RI; 4, DNA shown in lane 7 digested with *Eco*RI; 5, DNA shown in lane 8 digested with *Eco*RI; 6, DNA shown in lane 9 digested with *Eco*RI; 7, PCR product generated with primers M1 and M2 (see Fig. 1) from *F. heparinum* chromosomal DNA; 8, 1 μg PCR product generated with primers M1 and M2 from FIBX1; 9, 1 μg PCR product generated with primers M1 and M2 from pIBXF1.

resistance in *E. coli*. In addition, the gene was very small, only 250 bp, increasing the likelihood that it would be expressed and confer appreciable levels of antibiotic resistance in *F. heparinum*. The Tp-resistance (HT) cassette was constructed by fusing two PCR-generated DNA fragments consisting of the 830 bp *hepA* upstream region and a 250 bp *dhfrII* gene. Plasmid pIB20, containing the HT cassette, was introduced into *E. coli* but failed to confer Tp resistance, even when heparin was added to the growth medium. These data suggested that the *hepA* promoter was not functional in *E. coli*.

The final conjugative/integrative plasmid was constructed as shown in Fig. 1. The HT cassette and a randomly cloned 10 kbp *F. heparinum* chromosomal *Hin*dIII DNA fragment were added to pUC13-*oriT*. The features of the resulting plasmid, pIBXF1, include mobility conferred by pUC13-*oriT*, chromosomal integration through homologous recombination facilitated by the 10 kbp *F. heparinum* chromosomal DNA fragment, and selection conveyed by the HT cassette.

Introduction of pIBXF1 into *F. heparinum*

pIBXF1 was transferred into *F. heparinum* by conjugation as described in Methods. After more than 7 d incubation, colonies were isolated which displayed Km^r, Gm^r and Tp^r, as well as the ability to grow on FMH medium. One such strain was named FIBX1. The frequency of stable Tp^r transconjugants per recipient cell was approximately 10^{-10}.

To confirm that strain FIBX1 was a derivative of *F. heparinum*, it was purified to a single colony three times and further characterized by biochemical analysis. It was shown that FIBX1 could grow in FMH medium, was a Gram-negative bacterium, produced yellow pigment when grown on solid agar medium and had heparin and heparan sulfate degrading activity profiles similar to wild-type *F. heparinum* (data not shown). Together, these results confirmed that FIBX1 was a derivative of the wild-type *F. heparinum* strain.

It was possible that the Tp^r phenotype of FIBX1 was the result of a mutation, and/or selection of *F. heparinum* cells exhibiting a low level of Tp^r. To exclude this possibility and to confirm chromosomal plasmid integration, Southern analysis was performed. As shown in Fig. 3, *Hin*dIII/*Eco*RI digests of genomic DNA isolated from strain FIBX1 and plasmid pIBXF1 both hybridized to plasmid pIB21. Hybridization signals of 3·0 kbp and 1·1 kbp, corresponding to the vector pUC13-*oriT* and the HT cassette, respectively, were observed (Fig. 3b, lanes 2 and 3). No such signals were seen for genomic DNA isolated from wild-type *F. heparinum* (Fig. 3b, lane 1). These data clearly demonstrated the integration of plasmid pIBXF1 into the chromosome of strain FIBX1. More importantly, the successful recovery of Tp^r transconjugants indicated that the HT gene cassette could be used as a selective marker in *F. heparinum*.

The insertion site of plasmid pIBXF1 in the 10 kbp *Hin*dIII DNA fragment on the genome was confirmed by PCR and Southern analysis. If plasmid pIBXF1 inserted within the 10 kbp *Hin*dIII region, then the vector pIB21 should be flanked by the 10 kbp *Hin*dIII DNA fragments at both ends and in the same orientation. A pair of primers complementary to the ends of the randomly cloned 10 kbp *Hin*dIII insert from plasmid pIBXF1 (Fig. 1) should amplify the 4·1 kbp pIB21 vector from FIBX1 chromosomal DNA. As expected, DNA fragments corresponding to the size of pIB21 were generated from FIBX1 chromosomal DNA and plasmid pIBXF1 (Fig. 3a, lanes 8 and 9, respectively), but not from wild-type *F. heparinum* chromosomal DNA (Fig. 3a, lane 7). When these PCR products were digested with *Eco*RI, a 1·1 kbp band corresponding to the HT cassette and a 3·0 kbp band corresponding to plasmid pUC13-*oriT* were obtained (Fig. 3a, lanes 5 and 6), both of which hybridized to plasmid pIB21 (Fig. 3b, lanes 5 and 6). To exclude the possibility that plasmid pIBXF1 integrated in another region of the *F. heparinum* chromosome sharing homology with the 10 kbp *Hin*dIII DNA fragment, FIBX1 genomic DNA digested with *Hin*dIII was hybridized with that fragment but no additional signals were observed (data not shown). These results clearly demonstrate that FIBX1 carries an insertion of plasmid pIBXF1 within the 10 kbp *Hin*dIII DNA fragment of the *F. heparinum* chromosome.

Plasmid replication in *F. heparinum*

Two broad-host-range plasmids, pSUP106 (Frey & Bagdasarian, 1989), belonging to IncQ and pNJR12, an RSF1010 derivative containing a *Bacteroides* plasmid pB8-51 replicon (Maley *et al.*, 1992), and an *E. coli–C. johnsonae* shuttle plasmid, pCP11 (McBride & Kempf, 1996), were introduced into *F. heparinum* by conjugation but failed to yield selectable transconjugants (data not shown). We have demonstrated that the HT cassette is a functional selective marker and that plasmid DNA can be introduced by conjugation in *F. heparinum*. Therefore, it now became possible to define the replicative function of these plasmids in *F. heparinum*. Derivatives of pSUP106, pNJR12 and pCP11 were constructed by addition of an HT cassette and introduced into *F. heparinum* by conjugation. No stable Tp^r colonies were obtained. These plasmids lacked the ability to replicate in *F. heparinum*. An alternative broad-host-range plasmid, pBBR1Cm, was used to transform *F. heparinum* by electroporation but no Cm^r colonies were obtained (data not shown). To confer conjugative and selective functions to pBBR1Cm, the *mob*, Ap^r and HT elements were incorporated to form plasmid pIBXF2 as shown in Fig. 2. The plasmid was transferred into *F. heparinum* by conjugation. Several Tp^r colonies were obtained, which displayed characteristics similar to *F. heparinum*. The conjugation rate was similar to that obtained with the integrative plasmid pIBXF1. One of the new isolates was designated strain FIBX2.

Demonstration of the existence of plasmid DNA in FIBX2 was necessary to verify that the Tp^r phenotype was a result of episomal replication of pIBXF2 in *F. heparinum*. Plasmid DNA was isolated using the RPM method (see Methods), from a culture of FIBX2 grown in FMH medium, and analysed by electrophoresis. No plasmid DNA was visualized after ethidium bromide staining. Similar results were obtained when a highly concentrated plasmid preparation was subjected to restriction analysis (data not shown). However, when this DNA solution was electroporated into *E. coli*, Ap^r colonies were obtained. The digestion pattern of the plasmid DNA purified from these Ap^r colonies was identical to that of pIBXF2 (data not shown). These results confirmed the existence of plasmid pIBXF2 in strain FIBX2. The inability to visualize plasmid DNA from FIBX2 may be due to its existence as a low-copy-number plasmid in *F. heparinum*. Plasmid DNA from a FIBX2 culture was purified using Qiagen's low-copy-number plasmid purification method and analysed by electrophoresis (Fig. 4a, lane 3). No plasmid DNA band corresponding to pIBXF2 (Fig. 4a, lane 2) was seen. However, when the same DNA preparation was digested with *Eco*RI or *Xho*I (Fig. 4a, lanes 6 and 9, respectively), the restriction patterns were identical to that of pIBXF2 treated similarly (Fig. 4a, lanes 5 and 8). Hybridization of the restriction fragment with pIBXF2 identified them as the elements of the plasmid (Fig. 4b, lanes 5, 6, 8 and 9). The analysis also revealed the presence of a hybridization signal in the undigested pIBXF2 DNA

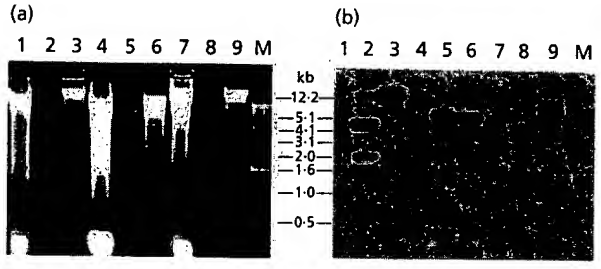

Fig. 4. Confirmation of the presence of pIBXF2 in strain FIBX2. (a) Ethidium bromide stained agarose gel. (b) Autoradiograph of Southern analysis of gel from (a) probed with plasmid pIBXF2. Lanes M, molecular mass markers – indicated in the middle (in kb); lanes 1, 4 and 7, 2 μg *F. heparinum* chromosomal DNA; lanes 2, 5 and 8, 1 μg pIBXF2 DNA from *E. coli*; lanes 3, 6 and 9, 15 μl DNA purified from strain FIBX2. Lanes 1–3, untreated DNA; lanes 4–6, *Eco*RI-digested DNA, lanes 7–9, *Xho*I-digested DNA.

preparation from FIBX2 (Fig. 4b, lane 3), that appeared to co-migrate with chromosomal DNA. The hybridization signals corresponding to the 2 kbp molecular mass marker (Fig. 4b, lanes 2, 5 and 8), which was not seen in the ethidium bromide stained gel, were probably due to partial plasmid degradation occurring during purification. *E. coli* transformed with pIBXF2 DNA yeilded Ap^r colonies and identical plasmid digestion patterns, as described previously (data not shown). The data from this study clearly show that pBBR1-based plasmids can replicate in *F. heparinum*.

Plasmid pIBXF2 is stably maintained in strain FIBX2. This was demonstrated by growing strain FIBX2 in the presence or absence of Tp and examining the Tp^r phenotype. Three hundred colonies from each group were tested and all of them showed resistance to Tp. Twelve colonies from the group growing in FH without Tp were cultured for isolation of plasmid DNA and *E. coli* transformation. All produced *E. coli* Ap^r colonies. In addition, 12 colonies from four of these 12 DNA preparations (three colonies each from four transformations) were grown and the plasmid DNA was isolated. Restriction analysis indicated that the plasmid DNA from these 12 isolates was identical to that of pIBXF2 (data not shown). These results, along with those described previously indicated that pIBXF2 could replicate in *F. heparinum* without selective pressure and be stably maintained.

Electroporation of pIBXF2

A method to introduce plasmid DNA into *F. heparinum* by electroporation was also developed. Typically, 20–50 *F. heparinum* Tp^r colonies could be obtained on selective plates within 7 d. As was the case with the transconjugants, undigested plasmid DNA isolated from these transformants could not be visualized by ethidium bromide staining of an agarose gel, but could be used to obtain Ap^r *E. coli* transformants.

DISCUSSION

We report here the creation of plasmid pIBXF1, a mobile plasmid with a randomly cloned 10 kbp *F. heparinum* DNA fragment and a selective marker consisting of the *dhfrII* (Tp^r) gene under the control of the *hepA* regulatory region. The plasmid was introduced into *F. heparinum* by conjugation, maintained through homologous recombination and transconjugants selected by acquisition of Tp resistance. Integration of plasmid DNA was confirmed at the molecular level. In addition, plasmid pIBXF2, a derivative of the broad-host-range plasmid pBBR1, has been shown to replicate in *F. heparinum*. This is the first report describing a genetic system for the introduction of heterologous DNA into this bacterium.

In our system, the rate of transconjugants produced with pIBXF1 was 10^{-10} per recipient. This low rate may be the result of a combination of factors including conjugation, homologous recombination and factors affecting conjugation efficiency. Surprisingly, a similar rate of transconjugant recovery was seen for pIBXF2, a derivative of the broad-host-range plasmid pBBR1, even though homologous recombination was no longer a required event. The possibility that a mutation in pFIBX2, either in the plasmid or in the host chromosome, that allowed maintenance of the plasmid in *F. heparinum*, was selected for during the conjugative process was considered. However, when pIBXF2 isolated from *E. coli* was reintroduced into *F. heparinum* or when pIBXF2 was introduced into the FIBX2 strain that had been cured of its plasmid, no increase in conjugation frequency was seen in either case (data not shown). The low efficiency of pIBXF2 conjugation in *F. heparinum* remains unexplained.

pIBXF2 replicated in *F. heparinum* irregularly. The plasmid copy number was significantly lower compared to pBBR1Cm as a high-copy-number plasmid in *E. coli* (Antonie & Locht, 1992), and 10 copies per cell described for *Brucella* species (Elizer *et al.*, 1995). In addition, the plasmid was somehow altered in strain FIBX2, as shown by it co-migrating with chromosomal *F. heparinum* DNA, while it retained a similar restriction pattern. Furthermore, its irregular plasmid form was also confirmed when examining plasmids recovered from *E. coli* after successive *F. heparinum* passages. Several large-size plasmids were observed (data not shown). Nevertheless, this study clearly demonstrates that pIBXF2 can replicate in *F. heparinum*. In a subsequent study, this plasmid system was used for high-level expression of both *cslA* and *cslB* in *F. heparinum* (F. Blain & others, unpublished results).

Plasmid pSUP106, a derivative of the broad-host-range IncQ plasmid RSF1010, was unable to replicate in *F. heparinum*. The pSUP106 derivative containing a *F. heparinum* functional selective marker, the HT cassette, also failed to generate *F. heparinum* transconjugants. This, along with other reported cases [*Bacteriodes* strains (Shoemaker *et al.*, 1986), *C. johnsonae* (McBride & Kempf, 1996), *Por. gingivalis* (Maley *et al.*, 1992) and *Pre. ruminicola* (Shoemaker *et al.*, 1991)] demonstrated the inability of plasmid RSF1010 and its derivatives to replicate in some Gram-negative bacteria. One possible explanation is that one or all of the *repABC* genes (Scherzinger *et al.*, 1984), involved in plasmid replication, are not functionally expressed in these organisms.

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